1980

Duration of Antibiotic Residues in Milk from Cows Treated via Intrauterine and Intramammary Infusion

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DURATION OF ANTIBIOTIC RESIDUES IN MILK FROM COWS TREATED VIA INTRAUTERINE AND INTRAMAMMARY INFUSION

BY

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A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science, Major in
Dairy Science, South Dakota
State University
1980

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ACKNOWLEDGMENTS

This thesis represents the concern, support, and assistance of many friends and family members abroad and in the United States without which this project could not have been successfully completed.

The author would like to express his deep appreciation to Dr. John G. Parsons for his encouragement, counsel, constructive criticism, and support throughout the duration of my course work.

A number of people rendered valuable assistance during the preparation of this thesis, and I would like to acknowledge their contributions here. I owe a special debt of gratitude to Dr. Kenneth R. Spurgeon, Dr. Roscoe J. Baker, and Mr. Myers J. Owens for their expertise, advice, cooperative spirit, and guidance that each of them brought to the initial proposal of this study.

I am deeply indebted to Mr. Al Schmidt and Laboratory Assistants and Land O'Lakes, Inc. for their assistance and cooperation in obtaining laboratory facility. I am grateful as well to Dr. William L. Tucker in his assistance and expertise in the statistical analysis of the data. Particular thanks go to Dr. Thomas M. Gilmore and Mark A. Wrucke for helping along with constructive criticism and comments.

Appreciation is expressed to Mr. Fenton C. Ludens and his milking crew for their help in treating the cows and reliable collection of milk samples in this study.

Many thanks are extended to graduate students, staff, and helpful secretaries for moral support and good times during my

stay at South Dakota State University.

To Marlys E. Moberg for her patience, conscientiousness, and tolerance for typing numerous drafts as well as the final copy of the manuscript. Her contribution was much greater than the simple word "typing" implies.

Thanks are due to Nenita F. Mendoza without whose unfailing confidence, patience, and immense good cheer the thesis would never have seen the light of day.

Finally, to my beloved family for their enduring love, support, encouragement, and for patience beyond all understanding and feeling truly blessed by their presence in my life, I respectfully dedicate this thesis.

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INTRODUCTION

Antibiotics have been employed by farmers and veterinarians for treatment of infectious cattle diseases for over 3 decades. The most common of these diseases in dairy cows is mastitis. Mastitis is defined by the National Mastitis Council (NMC) (49) as an inflammation of the mammary gland. It is the most prevalent and most costly disease of dairy cattle. It is estimated that at least 50% of all cows are infected with some form of mastitis at any one time in one or more quarters; cows in the average herd contract clinical mastitis one and one-half times per yr (49) which results in economic loss. According to the NMC, the average estimated total mastitis cost per cow per year is $161.00.

Penicillin alone or combined with other drugs has become the product of choice for treating many forms of infectious mastitis in the bovine. An estimated number of antibiotic treatments per case of mastitis is two (49). Therefore, using the NMC estimates and assuming 10 million cows in the United States, this places the total number of treatments in the United States at 30 million per year. In South Dakota, the estimated number of treatments is about 510,000 (49). With this level of use, the chance of penicillin or any other antibiotic contaminated milk reaching the market place exists.

Antibiotics, whether infused into the udder or injected intramuscularly, or intravenously, and more recently infused into the uterus, are secreted into the milk. The length of time needed for

the antibiotic residue to be completely absent from the milk following treatment is called withdrawal time. Withdrawal times from cows treated with antibiotic preparations have been determined and are listed on labels of most preparations.

Milk and milk products containing antibiotic residues are considered adulterated under the Food, Drug and Cosmetic Act of 1938. The Food and Drug Administration (FDA) has established a zero tolerance level of antibiotics in milk. Such products may present a significant hazard to health since small concentrations of antibiotics cause allergic reactions in some people and sensitivity reaction to antibiotics in others. Certain bacteria may develop resistance to antibiotics and become resistant to such treatment. Also, cultured products are difficult or impossible to make from milk containing antibiotic residues.

The Bacillus subtilis overnight disc assay method remains as the method of choice for monitoring antibiotic residues in producers' raw milk. The FDA recommends the Sarcina lutea cylinder plate method for determination of penicillin concentration in dry milk. The cylinder plate method is more sensitive to penicillin than the disc assay method (52) but takes 16 to 18 h to run. There is a need for a rapid method to assist the milk industry and milk regulatory agencies in detecting any level of antibiotics. There are new tests that have been developed recently which are simple, quick, and more sensitive. These tests are the Difco disc assay using Bacillus stearothermophilus, the agar diffusion test which

uses Bacillus stearothermophilus var calidolactis, and an enzyme immuno assay technique known as the Charm test.

One objective of this study was to measure the duration of antibiotics in milk from cows treated for uterine infections. Determinations for antibiotics were made by disc assay and the Charm test. Another objective was to compare the relative sensitivity between the Difco disc assay, the Charm test, and the disc assay for determination of penicillin in milk from cows treated via intramammary infusion.

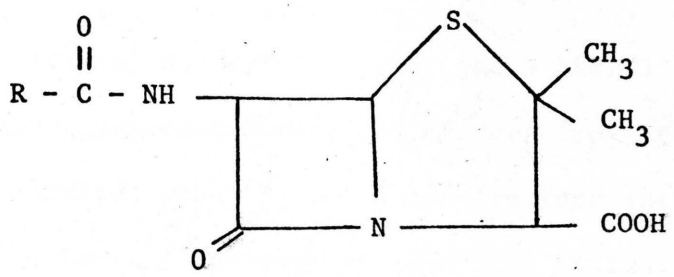
LITERATURE REVIEW

The success of antibiotics in therapy and related fields has made them one of the most important products of the modern drug industry today. An antibiotic is a chemical substance derived from or produced by various species of microorganisms, which is capable in small concentrations of inhibiting the growth of other microorganisms (32). Several antibiotics are currently used to combat mastitis, endometritis, or other infections in dairy cows. However, penicillin alone or combined with other antibiotics have been the drugs of choice and are used widely today. The discussion on the structure and mode of action of antibiotics has been limited to penicillin, dihydrostreptomycin, and nitrofurazone.

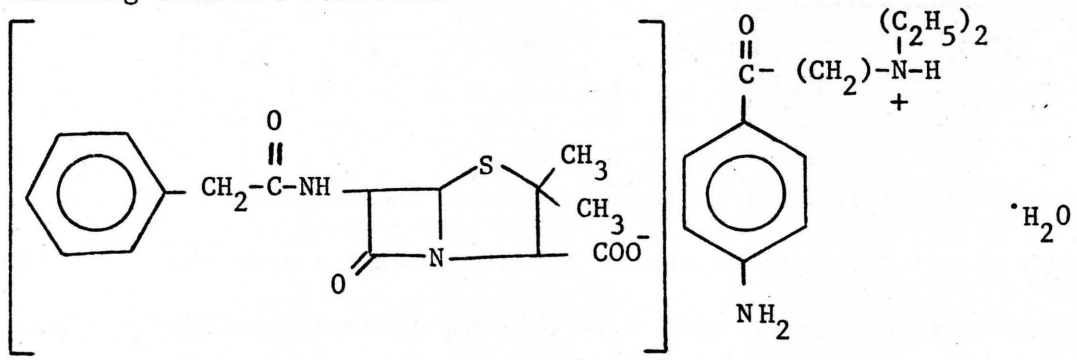
The antibiotic residues in milk from cows infused via intrauterine and intramammary routes has been discussed. A discussion on the development of the disc assay, the Difco disc assay, and the Charm test will also be given.

Chemical Structure and Mode of Action

Penicillin. Penicillin is a generic term for the entire group of natural and semisynthetic penicillins that have the same nucleus. It is produced by strains of Penicillium notatum and other species. These penicillins have a common chemical nucleus but differ in the nature of the acyl side chain (R) attached to this nucleus. The basic structure of penicillin is shown:



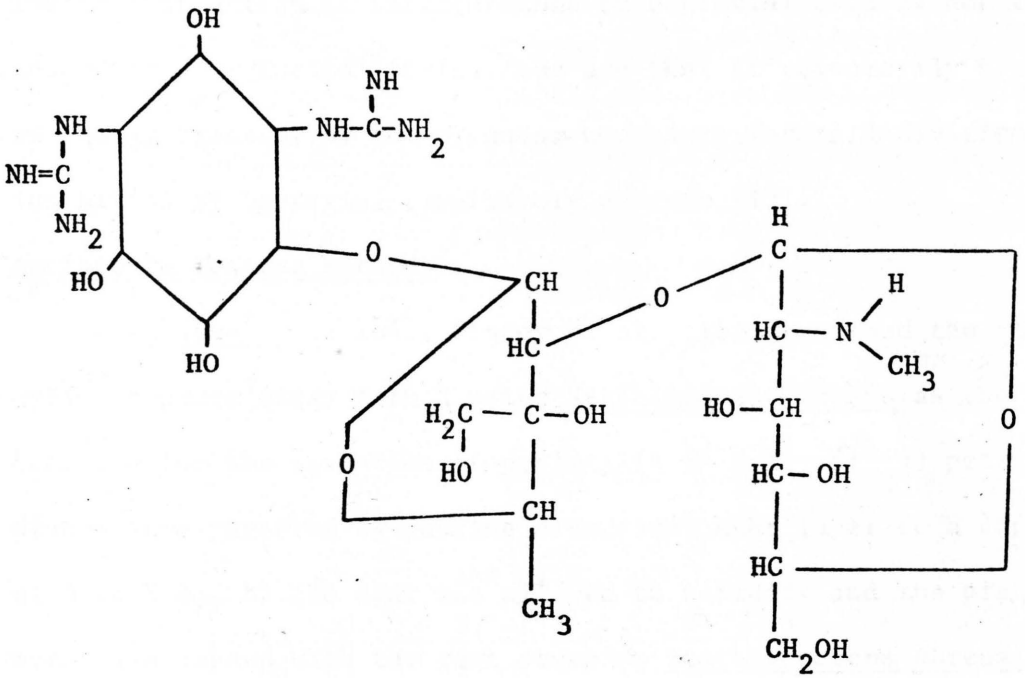
The (R) side chains are necessary for penicillin action and are introduced either chemically or biologically. Batchelor et al. (5) isolated a most useful chemical intermediate, β-aminopenicillanic acid, which was identified as a strong monobasic acid. Casida (8) described industrially produced penicillin as inherently unstable when in the free acid form and, therefore, should be prepared as salts or esters which are more stable. The penicillin used in this study was a semisynthetic penicillin called procaine penicillin G. This procaine salt of benzyl penicillin (penicillin G) has the following chemical structure:



Mode of action of penicillin is to inhibit cell wall synthesis (13, 32). Specifically, penicillin inhibits the bio-synthesis of the dipeptidoglycan that is needed to provide the strength and rigidity to the cell wall.

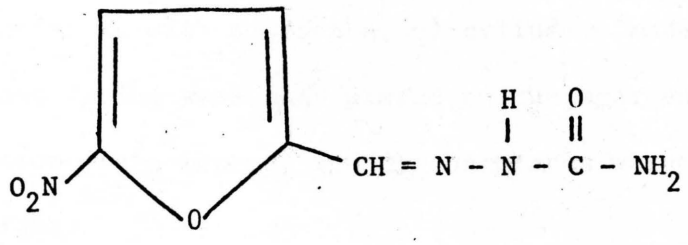
Dihydrostreptomycin. This synthetic antibiotic is composed of

striptidine, dihydrostreptose, and N-methyl-L-glucosamine joined by two glycosidic bonds. It differs from streptomycin only in that the aldehyde group on the middle residue (streptose) is reduced. The biological activity is identical to that of streptomycin (2, 22). The structure of dihydrostreptomycin is shown below:



This product was not known to be a product of microbial fermentation until 1957 (8) when a newly isolated streptomycetes strain, Streptomyces humidus, was found to produce it. Mode of action of dihydrostreptomycin is to inhibit protein synthesis (13, 22).

Nitrofurazone. It is one of the several hundred members of the nitrofuran compounds. It was first studied and reported in 1944 by Dodd and Stillman (14) as possessing good bacteriostatic and bactericidal properties. The structure for the nitrofurazone is shown:



The mode of action of nitrofurazone on bacterial cell is not readily understood. Principal indications are that it temporarily blocks an energy transfer by the organism necessary for cell division and inhibition of bacterial respiratory enzymes (13).

Antibiotic Testing Methods

Disc assay. In 1941, Florey et al. (18) described the first cylinder plate assay method using Staphlococcus aureus as the test organism for the detection of penicillin as follows: a) petri dishes were prepared by pouring a nutrient agar layer to a depth of 3 to 5 mm, b) the agar was allowed to solidify and the plates were then seeded with the test organism Staphylococcus aureus, by allowing a broth culture of the organism to flow over the surface of the agar and draining off the excess broth, c) the plates were dried (1 h) at 37 C in an incubator, d) cylinders made from short lengths of tubing (glass) were then placed and filled with the solution to be tested, and e) the plates were incubated at 37 C for 12 to 16 h. Foster and Woodruff (20) modified the method of Florey et al. (18) as follows: a) 100 ml cooled, melted agar was seeded with 0.1 ml of a 24 h nutrient broth culture of Staphylococcus aureus, b) the agar was uniformly measured into each plate,

13 ml and transferred with a pipette, c) cylinders made from short lengths of glass tubing were then placed on the agar and filled with the solution to be tested, and d) the plates were incubated at 37 C overnight.

Foster and Woodruff (20) cited several disadvantages of using Staphylococcus aureus as the test organism and the cylinders made from glass tubing. They described a method in which spores of Bacillus subtilis replaced Staphylococcus aureus as the test organism and filter paper discs replaced the cylinders. The B. subtilis culture has an advantage over that of S. aureus in that it is sensitive to a wide range of inhibitors. The paper discs are easier to apply than the cylinders. Loo et al. (39) developed a paper disc assay for streptomycin and described the advantage of using 12.7 mm filter paper discs instead of glass cylinders.

Welsh et al. (67) assayed milk samples from cows following parenteral administration of penicillin using a modified B. subtilis cylinder plate method of Foster and Woodruff (20). The plates were prepared by pouring 3 ml of agar medium seeded with spores of B. subtilis. Filter paper discs (7 mm diameter) were placed on the plates and 0.3 ml of the milk sample to be tested was added to each disc. The completed plates were incubated at 39 C and results were obtained after 4 h.

Silverman and Kosikowski (62) studied the disc assay method for detection of penicillin with the use of 7 mm and 12.7 mm filter paper discs. The 7 mm filter paper disc was saturated with

previously tested milk samples by holding the disc with tweezers and applying the milk with a micro pipette, whereas, the 12.7 mm filter paper disc was impregnated simply by dipping an edge in the milk until the entire disc was saturated by capillary action. The assay procedure using 7 mm filter paper disc had the ability to detect penicillin as low as 0.1 unit per ml in 4 to 6 h but was restricted to penicillin only. In order to destroy the naturally occurring inhibitory substances, the milk sample to be tested was heated for 5 min at 82 C (68, 69). The use of standard discs containing known concentrations of penicillin to serve as a check and identify penicillin as inhibitory substance was also described.

Cerny and Morris (9) developed a disc assay method which used two superimposed 12.7 mm filter paper discs. The 12.7 mm filter papers were saturated with the milk sample and placed one on top of the other on the surface of the agar. They reported the double disc method as being sensitive and precise to at least 0.01 units of penicillin per ml of milk while the 7 mm disc procedures lost precision below 0.1 unit per ml. They also found the double disc absorbed about ten times as much milk as a single 7 mm disc. They suggested the use of 6 ml of the seeded agar in a flat bottom sterile petri dish with incubation temperature of 37 C for 8 h or overnight (16 to 18 h) at room temperature. Siino et al. (61) compared the various discs suggested by other workers. They found that a single 12.7 mm disc was as sensitive for detecting penicillin as the double 12.7 mm disc. The 12.7 mm disc showed an advantage

over various discs (e.g. single or double 7 mm discs etc.), in that it was more sensitive, absorbed the milk samples uniformly, and the discs were very easy to handle due to their size.

Arret and Kirshbaum (4) developed a simplified rapid disc assay method which can detect a minimum concentration of 0.005 units of penicillin per ml of milk in 2 to 5 h. The procedure calls for the plates to be stored at 15 C for 3 to 5 days before being used and to remove the petri plates within 15 min of use. Johns (29) criticized this method as being less simple, less reliable, and less sensitive than claimed. Marth et al. (42) modified the method described by Arret and Kirshbaum (4) and developed a rapid disc assay method that could detect a concentration of 0.03 units per ml of penicillin in unheated milk at 37 C in 3 to 4 h.

The greatest sensitivity of antibiotics has been obtained with 6 ml penassay seed agar shown by the studies on the agar (medium #1) used in the disc assay test (3, 9, 30, 42). For best results in increasing sensitivity to detect penicillin residues, Marth et al. (42) suggested using of 0.1 to 1.0 x 10^6 spores of B. subtilis (ATCC #6633) per ml of agar and an incubation temperature of 32 C.

The Difco disc assay. This modified disc assay procedure developed by Difco Laboratories (PO Box 1058 A, Detroit, MI 48232) to detect the presence of antibiotic residues in milk provides both a color and an inhibition zone response. The test is based on the survival characteristics of bacterial spores. When performing the

test, Bacto-PM Indicator Agar and Bacto-Thermospore Suspension (B. stearothermophilus) are prepared and poured into flat bottom sterile petri plates. Using clean forceps, sterile discs are dipped into the milk sample and the disc is placed on the seeded agar surface. The plates are then incubated in inverted position in a single layer on the incubator shelf for 2 h and 40 min to 2 h and 50 min at 65 ± 1 C or for 3 h and 30 min to 3 h and 50 min at 56 ± 1 C. Finally, the plates are observed for color and zone response.

Igarashi et al. (26) indicated the potential usefulness of B. stearothermophilus as a test organism for detection of antibiotics. The organism was maintained by transfer on stock culture agar, with incubation at 55 C for 17 h or longer with interim storage at 4 C. Milk samples to be tested, as well as control samples, were pipetted in 10 ml quantities into test tubes. These samples were steamed for 7 min and cooled immediately in water at room temperature. Inoculum of 0.5 or 0.6 ml of the 17 h cultures was added to the samples. The tubes containing the milk plus culture were agitated to distribute the cells evenly and incubated in a water bath at 61 to 62 C for 20 min. After a 20 min period had elapsed, 0.5 ml of a 1% aqueous solution of Triphenyltetrazolium chloride (Eastman Kodak #6533) was added as an indicator. After addition of indicator, the tubes were held for an additional 10 to 20 min for color development and then was compared to the control tube known to be free of antibiotic residues. This procedure, with the use

of 17 h culture, detected 0.002 units of penicillin G per ml (26).

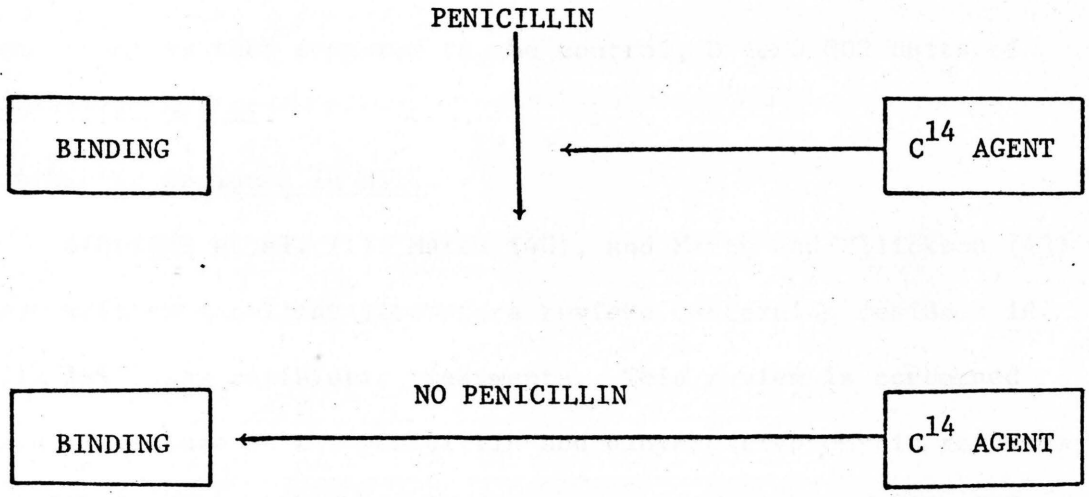
Mol (47) developed a rapid and sensitive test for the detection of penicillin in milk using petri plates. He used a culture of B. stearothermophilus var calidolactis which was incubated for 17 h at 63 C and stored in a refrigerator at 4 C. He detected 0.002 units of sodium penicillin G per ml in 2.5 to 4 h. Van Os et al. (66) and Kosikowski and Ledford (37) developed an agar diffusion test which uses B. stearothermophilus var calidolactis as a test organism. The rapid growth at elevated temperature and high sensitivity to penicillin and other antibiotics of B. stearothermophilus made it a valuable test organism. In 1970, the International Dairy Federation (IDF) published the disc plate method for the detection of antibiotics in milk using B. stearothermophilus as a test organism (27). This sensitive method required about 5 h to detect 0.0025 to 0.005 units of penicillin per ml (27). In 1977, Ouderkirk (53) described a modification of the IDF procedure. In a disc method using B. stearothermophilus var calidolactis, Ouderkirk (53) was able to detect 0.005 units of penicillin G per ml, 0.005 µg of ampicillin or cephapirin per ml, and 0.05 µg of cloxacillin per ml of milk in 3 to 4 h. Kaufman (34) also described a modification of the IDF procedure and was able to detect 0.004 unit of penicillin per ml of milk in 4 h on seeded plates previously stored for 7 days. The sensitivity was increased to 0.002 unit of penicillin per ml of milk when the plates were used the same day. The methods used by Ouderkirk (53), Kaufman (34), and IDF (27) do

not depend on a color response but utilize only the inhibition zone response for the detection of antibiotics.

Ginn et al. (21) evaluated the Difco disc assay which was an improvement over the method published by the IDF. The Difco test uses two parameters, zone of inhibition around the discs and color response to detect antibiotics. The concentration levels set for these two parameters to detect penicillin were 0.002 to 0.003 units per ml for color change from purple to yellow and 0.003 to 0.004 to as high as the 0.02 units per ml for zones of inhibition (48). Ginn et al. (21) were able to detect as low as 0.002 unit of penicillin per ml of milk on seeded plates stored for 1 wk; whereas, Morris (48) was unable to detect less than 0.004 unit of penicillin per ml of milk. Ginn et al. (21) further evaluated this assay procedure for reliability on rancid milk and stored seeded plates. The milk samples had Acid Degree Values of 2.7, 3.3, 4.1, and 6.1. The seeded plates were stored over a period of 3 wk. There was no noticeable effect on test results obtained from rancid milks, but there was a decrease in sensitivity on seeded plates stored over 1 wk. The minimum level of detectability had risen to 0.004 units and to 0.005 units of penicillin per ml upon 2 wk and 3 wk of storage, respectively.

The Charm test. This enzyme immuno assay technique was developed in 1978 by Dr. S. E. Charm of Tufts University. This quick (15 min), simple, and sensitive assay technique detects penicillin, streptomycin, and neomycin (10). Two reagents called A and B are

involved in the test. Reagent A contains the low level radioactive carbon 14 and reagent B contains a binder which absorbs carbon 14. The presence of any of the above drugs in the milk sample interferes with the binding of the carbon 14 (see diagram below)



In this test penicillin, streptomycin, and neomycin interfere with the binding of the carbon 14 agent (10). The lower the concentration of penicillin, the more carbon 14 agent is bound and shows higher reading on the analyzer. The test can detect concentrations as low as 0.002 units per ml in 15 min (10).

When performing the Charm test, reagents A and B are added one at a time to 5 ml of milk sample to be tested. After the reagents are incorporated into the milk sample, the sample is heated (3 min) and centrifuged (3 min). The supernate is decanted and the precipitate is resuspended in distilled water. The tube contents are poured into a small round aluminum planchet and placed on the hot

plate to evaporate the water. Finally, the planchet is inserted into the analyzer and the timer is set according to screening method chosen; 5, 8, 10 min, or time desired. The longer the time used the more accurate the result will be. A quantitative measure of the radioactivity from the carbon 14 is expressed as the count. This count is then compared to the control, 0 to 0.002 units of penicillin per ml.

Antibiotic Residues in Milk.

Albright et al. (1), Marth (40), and Marth and Ellickson (43) have written excellent literature reviews concerning residues in milk following antibiotic treatments. This review is concerned with literature on the penicillin and dihydrostreptomycin residues in milk from cows that received treatments via intrauterine infusion, and with penicillin residues in milk from lactating cows following intramammary infusion.

Intrauterine infusion. Literature concerning antibiotics in milk from lactating cows that have received an antibiotic treatment via intrauterine route is limited. A few reports have been published on the passage of antibiotics from the bovine uterus into the milk. Prouty (55) studied the presence of antibiotic residues in milk following intrauterine infusion with penicillin alone and with dihydrostreptomycin. Milk from cows that received 1×10^6 units of penicillin contained detectable amounts at 24 and 36 h after infusion, but the milk from cows that received a larger dosage of penicillin or penicillin in combination with

dihydrostreptomycin contained detectable amounts at 12, 24, 36, and 48 h. Kendrick and Pier (36) were unable to detect the presence of penicillin and dihydrostreptomycin in milk following the intrauterine infusion. So they concluded that it was not necessary to withhold the milk following treatment at the rate of 1×10^6 units per ml. Henningson et al. (24) examined milk from 25 dairy cows for penicillin, streptomycin, and Furacin residues following intrauterine infusion with these antibiotics. Milk samples were collected up to 96 h post-treatment, but no antibiotic residues were detected by the disc assay in the milk samples obtained from the treated cows.

Miller and Bergt (46) studied the differences between chelated and non-chelated oxytetracycline and the time required for their withdrawal from the milk following intrauterine administration. No residues were detected in the milk of any of the cows that were infused with the chelated oxytetracycline, but the cows that received the non-chelated oxytetracycline showed residues in the milk for 4 h through 24 h.

Kendrick (35) determined antibiotic residues in milk following uterine infusion. Penicillin (crystalline and procaine), terramycin, and dihydrostreptomycin were infused 3 to 6 h before milking. The milk from cows that received penicillin (crystalline) showed some residues only at the first milking. Furthermore, when the dose of penicillin was increased to 1×10^6 units per infusion, only crystalline penicillin appeared in the milk; none of the

other antibiotics were detected. Penicillin was found to be the most effective antibiotic for treatment of bacteria found in the uterus (35).

Intramammary infusion. When antibiotics were first used for the treatment of mastitis, they were commonly administered to dairy cows by means of intramammary infusion. Penicillin, perhaps, was the first of a series of antibiotics applied in mastitis therapy into infected quarters in an aqueous vehicle. Schalm and Casselberry (58), Thorp et al. (65), and Spencer et al. (63) found significant quantities of penicillin residues in milk samples 24 h after infusion with penicillin in an aqueous vehicle. Schalm and Casselberry (58) observed that the smaller the volume of milk excreted by quarters receiving treatment, the greater the efficacy of the penicillin infusions. Spencer et al. (63) employed mineral oil, peanut oil, and distilled water as carriers for penicillin. They found mineral oil to be more efficacious than aqueous solution or peanut oil as a carrier for penicillin in the therapy of bovine mastitis. The peanut oil suspension was inferior to the aqueous solution and in using aqueous vehicles several repeated doses were necessary to cure a case of mastitis (63). Mercer et al. (44) employed peanut oil suspension as a carrier for antibiotics and found that the vehicle exceeded the prescribed milk-out times. Foley et al. (19) compared a single infusion of 1×10^6 units in water and in a water-in-oil emulsion vehicle. Penicillin could be detected 72 h after infusion with water-in-oil emulsion vehicle. Foley et al.

(19) concluded that the persistence of penicillin was longer with water-in-oil preparations than with the aqueous carrier. They felt that this was due to the greater dispersion of the emulsion into the upper portion of the gland coupled with a slower excretion of the penicillin from the emulsion. Edwards and Haskins (15) and Jackson and Bryan (28) confirmed the findings of Foley et al. (19). Edwards and Haskins (15) also presented evidence of an inverse relationship between level of milk production and concentration of penicillin in milk.

Hueber et al. (25) studied an ointment or oil suspension and an aqueous solution as an intramammary mastitis treatment vehicle. They concluded that the aqueous infusions are superior to ointments or oil suspensions in therapy, due to greater and more even spreading and penetration within the mammary tissues. Schipper et al. (59) demonstrated that if rapidity of chemotherapeutic agent release is desired, the aqueous vehicle would be the choice. This is contrary to the Foley et al. (19) report. Blobel (6) studied the transfer of antibiotic (penicillin) from a treated quarter to an untreated quarter when penicillin in a water-in-oil emulsion vehicle was used. When 1×10^5 units and 3×10^5 units of penicillin were infused, penicillin concentrations in milk samples from untreated quarters ranged 0.005 to 0.010 units per ml to 0.005 to 0.060 units per ml, respectively. Penicillin persisted in the milk to treated quarters up to 120 h and in milk from untreated quarters up to 36 h following intramammary infusion. Blobel (6) also observed the correlation between penicillin levels

and yield of milk. Higher concentrations of penicillin persisted in the milk of low producers for longer periods than in the milk of high producers (6).

A study conducted by Cosgrove and Etgen (11) showed a crossover of antibiotic residues in four of 33 cows given intramammary infusions. One had residues in milk from untreated quarters for one milking, two for two milkings, and one for three milkings. The milk from the treated quarter of the cows contained antibiotic residues from four to 15 milkings. Evans and Stern (16) also studied the possibility of crossover in eight lactating cows. The cows were treated with maximum prescribed separate dosages of aqueous base and oil base procaine penicillin G over a 3 day period. They observed that penicillin in either an aqueous solution or in an oil suspension was transferred to untreated quarters in similar amounts, but the penicillin concentration in untreated and treated quarters was greatest in low producing animals. Ormiston et al. (51) discussed the possibility of antibiotic penetration from treated to untreated quarters using penicillin in aqueous solutions and in oil suspensions. They observed that the degree of penicillin penetration was greater with an aqueous solution than with an oil suspension. They also noted that the milk of two of the three mastitic cows showed no transfer of penicillin from treated to untreated quarters. The milk samples from all cows were free of penicillin by 72 h (51). Several workers (1, 6, 7, 17, 23, 51, 56, 57, 60, 70) substantiated the crossover of

antibiotic residues in milk from treated to untreated quarters in dairy cows. Johnson et al. (31) never detected any crossover of penicillin. Brown et al. (7) assumed that the diffusion of penicillin from treated to untreated quarters is via the venous system and secretory tissue rather than by direct infusion through the udder tissue. Rollins et al. (56) and Ziv et al. (70) indicated the possibility of some mechanism other than direct secretion from the blood stream being involved. A study conducted by Hawkins et al. (23) showed that the blood stream was not a sole mode of transfer of penicillin to the untreated quarters. Direct diffusion through the membrane is possible.

Today antibiotics commonly used for treatment of mastitis via intramammary infusion are carried by different vehicles, oil and water, which dictates different withdrawal times. All commercial antibiotic preparations have different withdrawal times which are approved by FDA; therefore, a wide range of withdrawal times of several antibiotics that were infused via intramammary infusion have been cited (6, 7, 11, 17, 31, 44, 45, 50, 56, 60, 65).

MATERIALS AND METHODS

Two separate studies were involved in this investigation. The first study was to measure for the persistence of antibiotics in milk from cows treated for uterine infections. The second study was to compare the relative sensitivity of three tests for detection of antibiotic residues in milk from cows treated via intramammary infusion.

Intrauterine Infusion

Thirty-one lactating Holstein dairy cows from the South Dakota State University dairy herd were treated by intrauterine infusion with one of three commercial antibiotic preparations. The cows were divided into two groups, one group consisted of cows with retained placentas and the other of cows without retained placentas. The antibiotic preparations used are designated as A, B, and C. The formulations for the three drugs (as prepared by Dr. R. N. Masson, 426 Third Street, Brookings, SD 57006) are as follows:

A = 6×10^6 units of procaine penicillin G with 0.13% methylparaben, 0.02% propylparaben, and 0.25% phenol as preservatives, 0.5% lecithin, 0.5% providone, 1% sodium citrate, not more than 0.01% sodium formaldehyde sulfoxylate, and 0.075% sodium carboxymethylcellulose.

B = 4×10^6 units of procaine penicillin G and 5 g of dihydrostreptomycin with 0.015% butylparaben, 0.37% sodium formaldehyde sulfoxylate, 2.0% procaine hydrochloride, and 0.25% phenol as preservatives, sodium citrate, 1.25%; lecithin, 0.25%; providone,

hydroxide.

C = Preparation B plus 200 ml of 0.2% w/v nitrofurazone and 4 g urea.

These antibiotics were chosen on the suggestion of a local veterinarian and with the aid of the literature cited (24, 35, 55). The cows were treated after the morning milking by SDSU herdsmen 3 days after parturition as outlined in Table 1.

TABLE 1. Experimental design for treatment of cows by intrauterine infusion.

Antibiotic preparation[a]	Number of cows	
	Retained placenta	Without retained placenta
A	5	6
B	5	6
C	4	5

[a]Antibiotic preparation described in text

The antibiotics were applied by inserting a 45 cm pipette into the uterus, making it possible to administer the drugs directly. The volume of the three drugs infused were different; A and B consisted of one 25 ml disposable syringe and C consisted of a 320 ml bottle. Treatment C was administered by using 25 ml sterilized disposable syringes. Composite milk samples were collected from the milking pre-treatment and from each milking up to 48 h after the treatment and stored at 4 C or frozen until analyzed for

antibiotic residues. The 48 h interval was selected based on observations by Henningson et al. (24), Kendrick (35), and Prouty (55).

Intramammary Infusion

Eight healthy lactating Holstein cows were treated via intramammary infusion with commercial antibiotic preparations. All cows were treated immediately after the morning milking by SDSU herdsmen. Two quarters were each infused once with one 10 ml plastet of the antibiotic preparation. The untreated quarters were each treated with a 10 ml plastet of distilled, sterile water. The treatment was applied as indicated in Table 2.

TABLE 2. Experimental design for treatment of cows by intramammary infusion.

Week	Cow number	Quarters treated[a]	
1	3530	LR	RF
	3589	LR	RF
	3656	RR	LF
	3671	LR	LF
2	3455	LR	LF
	3730	RR	LF
	3883	LR	LF
3	3665	RR	RF

[a]LR = Left rear, LF = Left front, RF = Right front, RR = Right rear

The formulation for the intramammary infusion contained 1 x 10^5 units of procaine penicillin G in oil with 50 mg chlorobutanol added as preservative. Milk samples were collected from each quarter from each milking up to 96 h after the treatment.

Assay Procedures

Sample testing. All milk samples collected from cows treated with intrauterine infusion were tested for antibiotic residues by using the Bacillus subtilis overnight disc assay method (41) and the Charm test[1]. Milk samples collected from cows treated with intramammary infusion were analyzed by Bacillus subtilis overnight disc assay method (41), the Difco disc assay[2] using Bacillus stearothermophilus, and the Charm test. Most milk samples were tested within 72 h of being taken; these samples were either stored at 4 C or held in a frozen state until analyzed. The samples for the Charm test were analyzed twice a week at a cooperating laboratory[3].

In order to determine if the zones of inhibition resulting from the milk samples were actually from penicillin, penicillinase-impregnated discs were placed near the disc with the test sample (41). If no zone of inhibition appeared around the penicillinase-impregnated disc but around the disc containing the test sample, penicillin was present. If there was no change in the zone of

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[1]The Charm Test, Penicillin Assays, Inc., Boston, MA 02111.

[2]Difco Laboratories, Detroit, MI 48232.

[3]Land O'Lakes Laboratory, Sioux Falls, SD 57104.

inhibition around both discs, an inhibitor other than penicillin was present. The inhibitory substances observed in milk samples from cows treated via intrauterine or intramammary infusion were confirmed to be penicillin. So the use of the penicillinase-impregnated disc was discontinued after the first 3 wk; however, from time to time the milk samples obtained from cows that received antibiotic treatment other than penicillin were checked.

Disc assay method. This overnight procedure was applied as outlined in Standard Methods for the Examination of Dairy Products (41). The Bacillus subtilis spore suspensions (ATCC 6633) were obtained from Difco Laboratories. A 1 ml vial of the spore suspension was added to 10 ml of monobasic potassium phosphate buffer (34 g of potassium dihydrogen phosphate in 500 ml of distilled water, adjust to pH 7.2 with 1 N sodium hydroxide, and made up to 1 liter mark with distilled water) to formulate a dilution of 1:11 (41).

Plates were prepared from a medium consisting of 1 ml of the 1:11 dilution of spore suspension in 99 ml of Antibiotic Medium No. 1 at a temperature of 50 to 55 C. Using an autoclaved syringe, 6 ml portions of seeded agar were transferred into the sterilized (dry heat) 100 mm diameter flat bottom glass petri dishes. The plates were used immediately after the agar was solidified.

Using clean, flamed forceps the edge of the sterile 13 mm blank disc was touched into the surface of a well mixed milk sample. The disc was allowed to absorb as much milk as possible by

capillary action and then touched to the rim of the flask to remove excess milk. The disc was placed immediately on the agar surface and touched gently with the tip of the forceps to assure proper contact with agar. Two discs were placed on each petri dish and care was taken to avoid placing the discs in the middle causing the zones of inhibition to overlap. After the completion of plating, the plates were inverted and incubated at 37 C for 12 to 14 h. After the incubation period, the diameter of each zone was measured to the nearest 0.5 mm.

The Charm test. This enzyme immuno assay technique was used according to directions from Penicillin Assays Inc. The test is briefly discussed in the text (page 14). The test calls for 5 min readings, however, preliminary data showed very low results. Also, due to the extreme variation and sensitivity of the test, a 10 min reading was performed on all the milk samples. The test also required a daily standard at a level of 0.025 units per ml of penicillin. The 0.025 units per ml standard comes with the kit in a tablet form which is dissolved in 100 ml antibiotic free pasteurized milk. From this milk a 0.005 units per ml standard was made by dilution. The zero standard was the antibiotic free pasteurized milk. This 15 min assay for penicillin, streptomycin, and neomycin (10) was performed at the Cooperating Laboratory 2 days a wk.

Difco disc assay. This assay technique using B. sterothermophilus was followed as outlined by Difco Laboratories with the exception of substituting one reagent in the test procedure. The

procedure calls for USP Purified Water whereas, distilled water was used. Plates were prepared by inoculating one ampule of the B. stearothermophilus spore suspension (ATCC 10149), given the trade name Bacto-Thermospore suspension PM, into 100 ml of Bacto-PM Indicator at a temperature of 60 to 65 C. Using an autoclaved syringe, 6 ml portions of seeded agar were transferred into the sterilized (dry heat) 100 mm diameter flat bottom glass petri dishes. The plates were used immediately after the agar was solidified.

Using clean, flamed forceps, the edge of the sterile 13 mm blank disc was touched into the surface of a well mixed milk sample. The disc was allowed to absorb milk bu capillary action as much as possible and then touched to the rim of the flask to remove excess milk. The disc was placed immediately on agar surface and touched gently with the tip of forceps to assure proper contact with agar. Two discs were placed on each petri dish and care was taken to avoid placing the discs in the middle causing the zone of inhibition to overlap. After the completion of plating, the plates were inverted and incubated in a single layer at 65 ± 1 C for 2 h and 50 min. Prior experiment showed longer incubation time required when the plates were stacked. In many cases the plates appeared readable at 2 h and 30 min showing a color change and a zone of inhibition, but were not read before the lapsed time suggested by the manufacturer. After the incubation period, the diameter of each zone was measured to the nearest 0.5 mm and the color change

of the disc was observed.

Preparation of standard curve. For determination of the penicillin residues in milk, a standard curve was prepared. Potassium penicillin G (USP convention, Inc., 12601 Twinbrook Parkway, Rockville, MD 20852) was used as a standard for penicillin. Eighty-one mg (128,000 units of penicillin) of the potassium penicillin G was weighed and dissolved in 100 ml of 1% phosphate buffer, pH 6.0 ±.1 (8.0 g monobasic potassium phosphate, 2.0 g dibasic potassium phosphate diluted to 1 liter with distilled water) (3, 38). The stock solution was diluted to give a concentration of 10 units penicillin per ml. This was further diluted in antibiotic free steamed 2% milk to obtain concentrations of 0.025, 0.05, 0.1, 0.2, 0.4, and 1.0 units pernicillin per ml (38). Figure 1 (31) shows the step by step dilution of the stock penicillin. The various concentrations of penicillin standards were tested by using the disc assay method used to determine the unknown samples. Zones of inhibition were measured and recorded as described previously. Finally, for each standard curve a linear regression equation was calculated by using:

$$y = mx + b$$

The value x represents the corrected zone diameters (mm) and the y value represents the standard concentration (log of units of penicillin per ml).

Statistical analysis. Statistical analysis for intrauterine infusion was by least squares analysis of variance for a 2 x 3 x 4

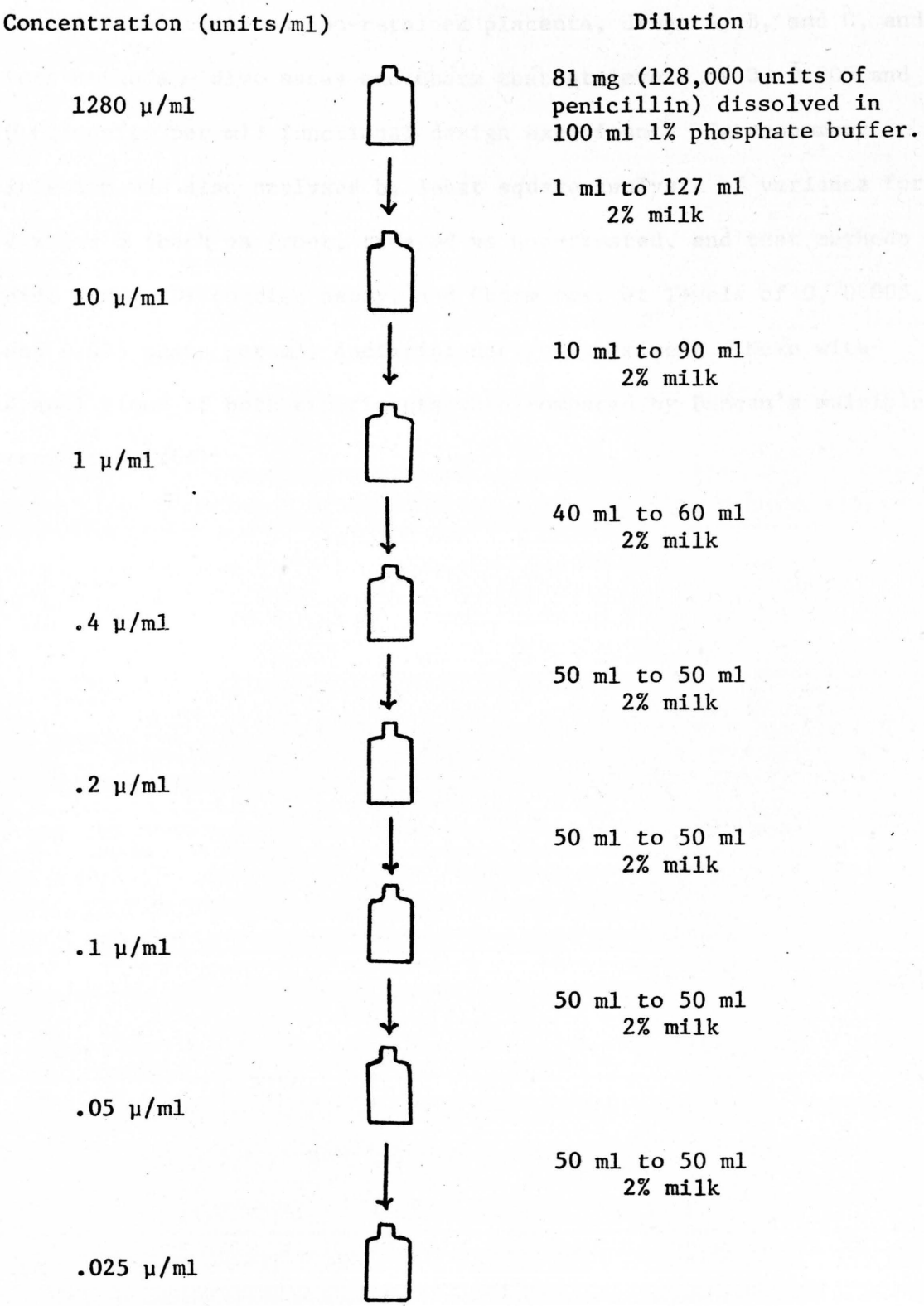

FIGURE 1. Preparation of penicillin standards.

(retained placenta vs non-retained placenta, drugs A, B, and C, and test methods - disc assay and Charm test at levels of 0, 0.005 and 0.025 units per ml) functional design experiment. Intramammary infusion was also analyzed by least square analysis of variance for 2 x 2 x 5 (back vs front, treated vs non-treated, and test methods - disc assay, Difco disc assay, and Charm test at levels of 0, 0.005, and 0.025 units per ml) factorial design experiment. Mean withdrawal times of both experiments were compared by Duncan's multiple range test (64).

RESULTS AND DISCUSSION

Antibiotic Residues in Milk Following Intrauterine Infusion.

The main objective of this study was to determine the presence and duration of the antibiotic residues in milk from cows treated via intrauterine infusion. On the average 15% or more milking cows undergo intrauterine medication (12). The infection and inflammation resulting from uterine contamination is called endometritis. It is usually treated by infusing antibiotic into the uterus, with the antibiotic treatments being made by the local veterinarian in most cases. The treatment is used to some degree in most of the states and widely used in California (35).

Thirty-one Holstein cows over a 6 mo period were involved in this experiment. Antibiotics were monitored by the standard disc assay and the new enzyme immuno assay technique called the Charm test (10, 41). The results of the Charm test from the cows that received antibiotics treatment via intrauterine route are shown in Tables 3 and 4. The numbers or counts that are shown under hours after infusion (0 to 48 h) and penicillin standards (0, 0.005, and 0.025 units per ml) are the average of the duplicate samples run on each sample. The values obtained from each milking were compared to the penicillin standard values of 0, 0.005, and 0.025 units to determine the amount of antibiotic residues each milk sample contained. For example, cow 3322 in Table 3 at the 48 h interval had a 10 min count of 814 which lies between 752 and 1215 for 10 min counts of penicillin standard. This means the

TABLE 3. Counts obtained by the Charm test for antibiotic residues following intrauterine infusion from cows without retained placenta.[a]

Type of drug[b]	Cow number	Hours after infusion[c]					Penicillin standards (μ/ml)		
		0	10	24	34	48	0	0.005	0.025
		(counts/10 min)[d]							
A	3322	1311	185	694	792	814	1215	752	360
	3523	935	539	904	953	1269	1179	879	460
	3555	1111	254	270	455	749	782	653	382
	3573	435	95	161	491	872	792	560	223
	3576	1141	384	1063	1033	1024	782	653	382
	3723	790	360	309	510	855	1215	752	360
B	3327	336	367	319	294	416	1179	879	460
	3382	434	418	119	278	304	792	468	243
	3627	1220	696	957	1098	1160	1179	879	460
	3650	1424	296	895	1156	1269	964	734	457
	3718	1448	174	288	440	882	792	560	223
	3730	1045	722	959	910	784	1179	877	460
C	3048	657	326	361	677	652	865	696	310
	3528	1155	562	605	818	1059	964	734	457
	3613	1249	207	930	1224	1414	1375	955	523
	3709	1228	457	623	799	971	1106	840	438
	3731	1273	602	748	901	1358	1215	752	360

[a]Cows were infused 3 days after parturition.

[b]Each drug consisted of A = 6,000,000 units of procaine penicillin G in aqueous suspension, B = 4,000,000 units of procaine penicillin G in 5 g dihydrostreptomycin, C = preparation B plus 200 ml of 0.2% (w/v) nitrofurazone solution and 4 g urea.

[c]Zero hour indicates pre-treatment milk samples.

[d]Each milk sample was counted 10 min in duplicate and the average was recorded as the final estimated value.

TABLE 4. Counts obtained by the Charm test for antibiotic residues following intrauterine infusion from cows with retained placenta.[a]

Type of drug[b]	Cow number	Hours after infusion[c]					Penicillin standards (μ/ml)		
		0	10	24	34	48	0	0.005	0.025
		(counts/10 min)[d]							
A	3255	1541	300	559	1260	1291	1076	890	404
	3416	1217	282	726	761	917	1076	890	404
	3511	895	217	309	806	1057	1215	752	360
	3578	1219	116	383	726	757	1215	752	360
	3755	798	94	324	394	1068	1076	890	404
B	3094	808	469	840	672	724	1179	879	460
	3468	111	169	744	1165	1263	1195	1047	619
	3474	1065	1222	1112	972	1060	1375	955	523
	3649	886	405	751	1063	1164	1370	874	501
	3665	1290	LA	1450	1398	1511	1258	878	442
C	3175	1102	791	1109	1049	1126	1076	890	404
	3405	863	348	469	446	832	1370	874	501
	3459	965	282	555	739	1039	868	659	351
	3674	1189	502	1043	1382	1507	792	468	243

[a]Cows were infused 3 days after parturition.

[b]Each drug consisted of: A = 6,000,000 units of procaine penicillin G in aqueous suspension, B = 4,000,000 units of procaine penicillin G in 5 g dihydrostreptomycin, C = preparation B plus 200 ml of 0.2% (w/v) nitrofurazone and 4 g urea.

[c]Zero hour indicates pre-treatment milk samples.

[d]Each milk sample was counted 10 min in duplicate and the average was recorded as the final estimated value. LA = Laboratory accident.

sample contained less than 0.005 units of penicillin but greater than 0 units. The penicillin standards with the same values were analyzed the same day. On these standards, extremely high variation was noted throughout the study. These variations can be shown between the penicillin standard values for cow 3555 and cow 3573 in Table 3, in which counts of 782, 653, and 382 and counts of 792, 560, and 223 were obtained with 0, 0.005, and 0.025 units per ml of penicillin, respectively. Therefore, these differences or variations obtained from the standards must not be taken as absolute values. The test lacks consistency on the digital readout whether the standards or the milk samples to be tested are used. The penicillin standards (0, 0.005, and 0.025 units per ml) were divided into three categories, (+) = <0.005, (++) = 0.005 to 0.025, (+++) = >0.025 units per ml so that a comparison of the two tests could be established (Tables 5 and 6).

Results of milk residues of the antibiotics (A, B, and C) infused into the uterus as determined by the disc assay and the Charm tests are shown in Tables 5 and 6. The pre-treatment milk samples which are denoted by zero hour interval, showed some antibiotic residues. Fifteen out of thirty-one milk samples showed some antibiotic residues by the Charm test method, whereas, antibiotic residues in only one of the milk samples (cow 3468) was detected by the disc assay. Those cows which showed antibiotic residues may have received a dry cow treatment or the milk may have contained substances which interferred with the Charm test. All the

TABLE 5. Antibiotic residues in milk following intrauterine infusion of cows without retained placenta.[a]

Type of drug[b]	Cow number	Type of assay[c]	Hours after infusion[d]				
			0	10	24	34	48
			(units/ml)[e]				
A	3322	CT	-	+++	++	+	+
		DA	0	0.024	0	0	0
	3523	CT	+	++	+	+	-
		DA	0	0	0	0	0
	3555	CT	-	+++	+++	++	+
		DA	0	0.020	0.020	0	0
	3573	CT	++	+++	+++	++	-
		DA	0	0.10	0.031	0	0
	3576	CT	-	++	-		
		DA	0	0	0		
	3723	CT	+	++	+++	++	+
		DA	0	0.020	0.021	0	0
B	3327	CT	+++	+++	+++	+++	+++
		DA	0	0	0	0	0
	3382	CT	++	++	+++	++	++
		DA	0	0	0.024	0	0
	3627	CT	-	++	+	+	+
		DA	0	0	0	0	0
	3650	CT	-	+++	+	-	
		DA	0	0.029	0	0	
	3718	CT	-	+++	++	++	-
		DA	0	0.028	0	0	0
	3730	CT	+	++	+	+	+
		DA	0	0	0	0	0
C	3048	CT	++	++	++	++	++
		DA	0	0	0	0	0
	3528	CT	-	++	++	+	-
		DA	0	0	0	0	0
	3613	CT	+	+++	++	+	+
		DA	0	0.065	0	0	0
	3709	CT	-	++	++	++	+
		DA	0	0	0.031	0	0
	3731	CT	-	++	++	+	-
		DA	0	0.16	0.019	0	0

[a]Cows were infused 3 days after parturition.

[b]Each drug consisted of: A = 6,000,000 units of procaine penicillin G in aqueous suspension, B = 4,000,000 units of procaine penicillin G in 5 g dihydrostreptomycin, C = preparation B plus 200 ml of 0.2% (w/v) nitrofurazone solution and 4 g urea.

[c]CT = Charm test, DA = Disc assay.

[d]Zero hour indicates pre-treatment milk samples.

[e]Zero indicates no detectable antibiotic residue; (+) = <0.005, (++) = 0.005 to 0.025, (+++) = >0.025, (-) = negative test.

TABLE 6. Antibiotic residues in milk following intrauterine infusion of cows with retained placenta.[a]

Type of drug[b]	Cow number	Type of assay[c]	Hours after infusion[d]				
			0	10	24	34	48
			(units/ml)[e]				
A	3255	CT	-	+++	++	-	-
		DA	0	0.037	0	0	0
	3416	CT	-	+++	++	+	++
		DA	0	0.030	0	0	0
	3511	CT	+	+++	+++	+	+
		DA	0	0.022	0.021	0	0
	3578	CT	-	+++	++	++	+
		DA	0	0.038	0	0	0
	3755	CT	++	+++	+++	+++	+
		DA	0	0.124	0.029	0.018	0
B	3094	CT	++	++	++	++	++
		DA	0	0	0	0	0
	3468	CT	+++	+++	++	+	-
		DA	0.495	0.132	0	0	0
	3474	CT	+	+	+	+	+
		DA	0	0	0	0	0
	3649	CT	+	+++	++	+	+
		DA	0	0.025	0	0	0
	3665	CT	-	LA	-	-	
		DA	0	LA	0	0	
C	3175	CT	-	++	-	+	-
		DA	0	0	0	0	0
	3405	CT	++	+++	+++	+++	++
		DA	0	0.033	0	0	0
	3459	CT	-	+++	++	+	-
		DA	0	0.020	0	0	0
	3674	CT	-	+	-	-	
		DA	0	0	0	0	

[a]Cows were infused 3 days after parturition.

[b]Each drug consisted of: A = 6,000,000 units of procaine penicillin G in aqueous suspension, B = 4,000,000 units of procaine penicillin G in 5 g dihydrostreptomycin, C = preparation B plus 200 ml of 0.2% (w/v) nitrofurazone solution and 4 g urea.

[c]CT = Charm test, DA = Disc assay.

[d]Zero hour indicates pre-treatment milk samples.

[e]Zero indicates no detectable antibiotic residue, (+) = <0.005, (++) = 0.005 to 0.025, (+++) = >0.025, (-) = negative test, LA = Laboratory accident.

milk samples that were monitored by the Charm test showing (+++) were also positive by the disc assay with the exception of those from cow 3327 without retained placenta and cow 3405 with retained placenta. This could have been due to the interference of antibiotics other than penicillin with the binding of carbon-14 agent. The samples were run at least three times with both testing methods and no definite conclusions could be drawn except that there was a substance interferring with the binding reagent. It was also noted that the two testing methods correlated vey well.

No significant difference ($P>.05$) was observed when the mean lengths of time each antibiotic stayed in the milk sample (31.81 h for A, 31.32 h for B, and 28.32 h for C) as determined by the disc assay and the Charm test at three levels were compared. Table 7 shows the individual drug mean withdrawal time. For statistical purposes the withdrawal times were estimated beyond 48 h. A considerable variation occurred in the withdrawal time of each drug; this could have been due to the variation within each cow. Also, no statistical differences ($P>.05$) were noted between the withdrawal times of cows with or without retained placenta. Prouty (55) detected some antibiotic residues up to 48 h in the milk samples from cows that received approximately 7×10^6 units per ml of penicillin and 7×10^6 units of penicillin plus 5.5 mg of dihydrostreptomycin. Those cows that received 1×10^6 units of penicillin showed detectable amounts of residues in the milk at 24, 36, and 48 h (55). On the other hand Kendrick (35) could only detect penicillin alone at 12 h interval

TABLE 7. Withdrawal times of antibiotics in milk by the disc assay and the Charm test following intrauterine infusion.

Item[a]	Type of antibiotic assay			
	Disc assay	Charm test		
		0.025	0.005	0
Mean withdrawal time				
Drug A (h)	26.07	26.07	40.03	51.10
B (h)	12.83	18.91	41.47	52.10
C (h)	15.20	11.40	38.30	48.40
All drugs (h)	18.03[b]	18.79[b]		
Range (h)	10-24	10-24		
No. of milkings	1-2	1-2		
All drugs (h)		18.79[c]	39.93[d]	50.53[e]
Range (h)		10-24	24-48	24-48+
No. of milkings		1-2	2-4	2-4+

[a] A = 6 x 10^6 units of procaine penicillin G in aqueous suspension, B = 4 x 10^6 units of procaine penicillin G in 5 g dihydrostreptomycin, C = preparation B plus 200 ml of 0.2% (w/v) nitrofurazone solution and 4 g urea.

[bcde] ($P<.05$) Means with the same superscript are not significantly different.

and with increased dosage at 24 h interval.

The results shown in Table 7 also show the withdrawal time of the antibiotics following intrauterine infusion as determined by the two testing methods. A more meaningful interpretation can be made by chaning the time interval into the number of milkings it would take for complete removal of the antibiotic from the milk. The disc assay and the Charm test for all drugs showed a good correlation at the 0.025 units per ml level, 18.03 and 18.79 h, respectively. The difference in length of time all drugs were detected by the Charm test at three levels (18.79 h, 39.93 h, 50.53 h) were statistically significant ($P<.05$). It was evident that the Charm test detected antibiotic residues for longer time and was more sensitive than the disc assay.

Table 7 shows that the Charm test at 0.005 and 0 level detected antibiotic residues after intrauterine infusion for two more milkings than the disc assay. The comparison of the two methods was done at three levels for the Charm test and one level for the disc assay. The results from the analysis of variance are listed in Appendix Table 1. The data show that the difference between the two testing methods was statistically significant ($P<.01$).

Results obtained in this study suggested withholding milk samples obtained from the treated animals at least for four milkings or 48 h. Since many farmers do not withhold milk following intrauterine infusion, this may be a reason for the increasing incidence of antibiotics in the milk supply. If a cow is treated for any infection with any antibiotic, including antimastitis preparations,

the milk should be withheld for a time according to the label or at least for 48 h. A larger number of positive samples would be antibipated if the Charm test or any other new, more sensitive testing method is used to assay milk samples instead of the disc assay method. The Charm test is more sensitive, quicker (15 min), and simpler than the disc assay method in that no specialized microbiological techniques are required. However, the Charm test shows variation upon replication.

Antibiotic Residues in Milk Following Intramammary Infusions.

Antibiotic residue testing is important for incoming bulk tank raw milk where a simple, rapid, and sensitive test is necessary. The second objective of this study was to compare the relative sensitivity of three tests for detection of antibiotic residues in milk from cows treated via intramammary infusion. The three antibiotic tests used were the standard overnight disc assay, the modified and approved (July 1, 1980) method of the disc assay called the Difco disc assay, and the Charm test. The relative sensitivities of the disc assay, Difco disc assay, and Charm test for penicillin were: 0.025 units per ml, 0.002 units per ml, and less than 0.002 units, respectively (4, 10, 21, 30).

Table 8 shows the data obtained from the Charm test following intramammary infusion. The numbers or counts that are shown under milking intervals (10 to 96 h after infusion) are the average of the duplicate samples. The penicillin standard values are given following one or more cows. Standards showing same values

TABLE 8. Counts obtained by the Charm test for penicillin residues in milk from cows following intramammary infusion.[a]

Cow number	Sample collected from quarters[b]	Penicillin stds[c]	Hours after infusion							
			10	24	34	48	58	72	82	96
			(count/10 min)[d]							
3530	Right rear		756	1337	0	0				
	Left front		957	1052	0	0				
	Left rear*		65	61	109	399	723	1150	0	0
	Right front*		82	98	111	316	965	1053	1080	0
3589	Left front		1032	1179	0	0				
	Right rear		1002	1224	0	0				
	Left rear*		82	89	179	886	763	1005	1260	0
	Right front*		79	63	66	203	236	741	1316	0
3656	Right front		957	1006	0	0				
	Left rear		824	270	1325	0	0			
	Left front*		64	70	83	907	1145	987	1095	0
	Right rear*		58	78	116	390	1161	1259	0	0
3671	Right front		LA	1074	0	0				
	Right rear		67	1097	0	0				
	Left rear*		64	82	347	988	1480	0	0	
	Left front*		58	174	158	678	1443	0	0	
		0.000	1375	1090	1090	1033	1200	1076	1076	
		0.005	955	885	885	786	952	890	890	
		0.025	523	541	541	633	549	404	440	
3665	Left rear		716	1001	0	0				
	Left front		707	868	1042	0	0			
	Right rear*		61	90	170	521	868	796	945	0
	Right front*		68	74	174	379	799	956	0	0
		0.000	914	914	914	914	914	914	914	
		0.005	608	708	708	708	708	708	708	
		0.025	502	502	502	502	502	502	502	

Table continued on next page

TABLE 8. continued.

Cow number	Sample collected from quarters[b]	Penicillin stds[c]	Hours after infusion							
			10	24	34	48	58	72	82	96
			(count/10 min)[d]							
3455	Right rear		916	929	1121	LA	884	1042	0	0
	Right front		1061	1084	1103	LA	1028	0	0	
	Left rear*		63	78	307	625	834	1060	0	0
	Left front*		96	88	181	293	662	838	993	954
3730	Left rear		754	836	1118	1345	0	0		
	Right front		655	691	1373	0	0			
	Left front*		66	73	559	642	1173	0	0	
	Right rear*		71	75	620	1060	0	0		
3883	Right rear		1120	1185	1149	1080	0	0		
	Right front		1039	1195	1323	0	0			
	Left front*		79	323	1222	1349	0	0		
	Left rear*		64	156	1022	1219	0	0		
		0.000	1258	1258	1258	1029	1029	1029	1029	1029
		0.005	878	878	878	847	847	847	847	847
		0.025	442	442	442	374	374	374	374	374

[a]Each milk sample was counted 10 min in duplicate and the average was recorded as the final estimated value.

[b]Detectable crossover occurred between treated and non-treated quarters.

[c]Units of penicillin added to pasteurized milk free of any antibiotic residue.

[d]Zero indicates no detectable antibiotic residue, LA = laboratory accident.

*Treated quarters.

under each milking interval denote same day sample analysis. The samples were collected from treated and untreated quarters. The cows were treated at various times in a group of four, one, and three as indicated in Table 8. Milk samples obtained from left front quarter of cow 3455 showed antibiotic residues up to 96 h which passed the recommended withdrawal time by three milkings (60 h). The extreme variation of the Charm test on the standards or milk samples to be tested were seen in this study. Therefore, the values obtained from standards in this study also must not be considered as absolute values. The penicillin standards (0, 0.005, and 0.025 units per ml) were divided into three categories: 1) (+) = <0.005, 2) (++) = 0.005 to 0.025, and 3) (+++) = >0.025 units so that a comparison of the tests could be established (Table 9).

Levels of penicillin residues in the milk from each quarter as determined by the disc assay, Difco disc assay, and Charm test methods are given in Table 9. After two quarters were infused, detectable amounts of penicillin in the milk from untreated quarters (crossover) were found. The crossover of antibiotics from treated to untreated has been reported in numerous reports and this finding agrees with those studies (1, 6, 7, 17, 23, 51, 56, 57, 60, 70). Similarly there are reports that this transfer to untreated quarters does not occur (31, 33). Penicillin residues were detected in the milk from untreated quarters of eight cows at 10 h post treatment by the disc assay and the Difco disc assay and at 10, 24, and 34 h by the more sensitive Charm test. There were

TABLE 9. Procaine penicillin G in milk following intramammary infusion.[a]

Cow number	Sample collected from quarters[b]	Type of assay[c]	Hours after infusion							
			10	24	34	48	58	72	82	96
			(units/ml)[d]							
3455	Right rear	CT	+	+	+	LA	+	-	-	
		DA	0	0	0	0	0	0	0	
		DDA	0	0	0	0	0	0	0	
	Right front	CT	+	+	+	LA	-	-		
		DA	0	0	0	0	0	0		
		DDA	0	0	0	0	0	0		
	Left rear*	CT	+++	+++	+++	++	++	-	-	
		DA	0.925	0.102	0.029	0	0	0	0	
		DDA	8.450	1.73	0.124	0.053	0	0	0	
	Left front*	CT	+++	+++	+++	+++	++	++	+	+
		DA	0.966	0.179	0.064	0.027	0	0	0	0
		DDA	7.290	2.40	0.39	0.135	0	0	0	0
3530	Left front	CT	+	+	-	-				
		DA	0	0	0	0				
		DDA	0.01	0	0	0				
	Right rear	CT	++	-	-					
		DA	0.025	0	0					
		DDA	0.131	0	0					
	Right front*	CT	+++	+++	+++	+++	+	+	-	-
		DA	3.77	0.505	0.556	0.072	0	0	0	0
		DDA	27.56	4.160	2.87	0.250	0.058	0	0	0
	Left rear*	CT	+++	+++	+++	+++	++	-	-	
		DA	3.57	1.57	0.237	0.037	0	0	0	
		DDA	26.98	8.96	1.37	0.198	0.063	0	0	
3589	Left front	CT	+	-	-					
		DA	0	0	0					
		DDA	0.111	0	0					
	Right rear	CT	+	-	-					
		DA	0.036	0	0					
		DDA	0.065	0	0					
	Left rear*	CT	+++	+++	+++	+	++	+	-	-
		DA	3.04	0.448	0.090	0	0	0	0	0
		DDA	22.20	2.970	0.402	0.038	0	0	0	0
	Right front*	CT	+++	+++	+++	+++	+++	++	-	-
		DA	5.39	1.49	1.11	0.111	0.083	0	0	0
		DDA	40.24	10.42	3.61	0.554	0.394	0	0	0

Table continued on next page

TABLE 9. continued.

Cow number	Sample collected from quarters[b]	Type of assay[c]	Hours after infusion							
			10	24	34	48	58	72	82	96
			(units/ml)[d]							
3656	Right front	CT	+	+	-	-				
		DA	0	0	0	0				
		DDA	0.745	0	0	0				
	Left rear	CT	++	+++	-	-				
		DA	0	0	0	0				
		DDA	0.071	0	0	0				
	Left front*	CT	+++	+++	+++	+	+	+	-	-
		DA	16.06	0.217	0.123	0	0	0	0	0
		DDA	91.52	3.140	1.658	0	0	0	0	0
	Right rear*	CT	+++	+++	+++	+++	+	-	-	
		DA	5.51	0.778	0.162	0	0	0	0	
		DDA	44.84	6.760	1.490	0	0	0	0	
3665	Left rear	CT	+	-	-					
		DA	0	0	0					
		DDA	0	0	0					
	Left front	CT	++	+	-	-				
		DA	0	0	0	0				
		DDA	0.047	0	0	0				
	Right rear*	CT	+++	+++	+++	++	+	+	-	-
		DA	7.11	0.666	0.111	0	0	0	0	0
		DDA	17.13	1.97	0.627	0.065	0	0	0	0
	Right front*	CT	+++	+++	+++	+++	+	-	-	
		DA	6.07	0.647	0.126	0	0	0	0	
		DDA	15.71	2.29	0.631	0.054	0	0	0	
3671	Right front	CT	LA	+	-	-				
		DA	0.032	0	0	0				
		DDA	0.098	0	0	0				
	Right rear	CT	+++	-	-					
		DA	0.054	0	0					
		DDA	0.168	0	0					
	Left rear*	CT	+++	+++	+++	+	-	-		
		DA	4.11	1.05	0.039	0	0	0		
		DDA	32.42	4.78	0.288	0	0	0		
	Left front*	CT	+++	+++	+++	++	-	-		
		DA	2.85	0.51	0.072	0	0	0		
		DDA	17.13	7.53	0.569	0.046	0	0		

Table continued on next page

TABLE 9. continued.

Cow number	Sample collected from quarters[b]	Type of assay[c]	Hours after infusion 10	24	34	48	58	72	82	96
			(units/ml)[d]							
3730	Left rear	CT	++	++	+	-	-			
		DA	0	0	0	0	0			
		DDA	0	0	0	0	0			
	Right front	CT	++	++	-	-				
		DA	0	0	0	0				
		DDA	0	0	0	0				
	Right rear*	CT	++	+++	++	-	-			
		DA	0.551	0.128	0	0	0			
		DDA	8.00	2.440	0.071	0	0			
	Left front*	CT	+++	+++	++	++	-	-		
		DA	0.93	0.181	0.030	0	0	0		
		DDA	5.36	3.77	0.091	0	0	0		
3883	Right rear	CT	+	+	+	-	-			
		DA	0	0	0	0	0			
		DDA	0	0	0	0	0			
	Right front	CT	+	+	-	-				
		DA	0	0	0	0				
		DDA	0	0	0	0				
	Left front*	CT	+++	+++	+	-	-			
		DA	0.242	0	0	0	0			
		DDA	1.860	0.745	0	0	0			
	Left rear*	CT	+++	+++	+	-	-			
		DA	0.563	0.052	0	0	0			
		DDA	5.810	0.215	0.048	0	0			

[a]Each infusion consisted of 100,000 units of procaine penicillin G in an oil based preparation.

[b]Detectable crossover occurred between treated and non-treated quarters.

[c]CT = Charm test, DA = Disc assay, DDA = Difco disc assay.

[d]Zero indicates no detectable antibiotic residue; (+) = <0.005, (++) = 0.005 to 0.025, (+++) = >0.025, (-) = negative test, LA = laboratory accident.

*Treated quarters.

differences in sensitivity of the assay methods. Out of the eight cows involved, milk samples from cow numbers 3530, 3589, and 3671 showed crossover by the disc assay. Milk samples from cow numbers 3530, 3589, 3656, 3665, and 3671 showed crossover by the Difco disc assay. However, crossover was detected in milk samples from all eight cows by the Charm test. It was also noted that the Charm test and the Difco disc assay detected penicillin residues in milk from both untreated quarters in most of the cows that were involved, whereas, the disc assay detected crossover in only one of the cows (cow 3671). Recommended withdrawal time of five milkings (60 h) was not exceeded when milk was monitored either by the disc assay (48 h) or the Difco disc assay (58 h) or the Charm test at 0.025 level (48 h). However, antibiotic residues detected by the Charm test at 0 and 0.005 level exceeded the recommended withdrawal time by three milkings (72, 82, and 96 h).

The data in Table 10 show that the Charm test at three levels of sensitivity detected penicillin residues after intramammary infusion for a longer period of time than the disc assay or the Difco disc assay methods, 30.75, 42.94, and 53.0 h versus 30.81 and 36.63 h, respectively. The difference in length of time penicillin was detected was statistically significant ($P<.05$). No statistical difference ($P>.05$) in mean withdrawal time was noted when comparing the disc assay with the Difco disc assay or the Charm test at 0.025 level. In fact, a good correlation could be seen between the disc assay and the Charm test at 0.025 level. It is evident that the

Charm test detected for longer time and was more sensitive than the disc assay or Difco disc assay.

A considerable variation occurred in the range for withdrawal of penicillin given via intramammary infusion (Table 10). For the intramammary penicillin preparation used in this study 60 h or five milkings was the manufacturer's recommended withdrawal time. As can be seen in Table 10, the manufacturer's recommended withdrawal time of five milkings was exceeded by three milkings when the milk from cows that had received intramammary infusion was checked with the Charm test. Plastridge (54) reported that it is not unusual to find milk positive for antibiotics beyond the recommended hours post treatment milk disposal period. Since composite milk samples (all four quarters) were not collected; it is not known if these residues would have persisted as long. This leads to a question of what would happen if a milk sample from a treated cow were added to the milk in the bulk tank? Antibiotic diluted in the milk supply probably could not be detected by current approved methods.

The data in Table 10 show that the Charm test at 0 and 0.005 level detected antibiotic residues after intramammary infusion for longer periods of time than the disc assay or the Difco disc assay. The comparison for the three methods was done at three levels for the Charm test and one level for the disc assay and the Difco disc assay. The results from the analysis of variance are listed in Appendix Table 2. The data show that the differences between treated and non-treated quarters and the testing methods are

statistically significant (P<.01) indicating crossover between treated and untreated quarters and the Charm test was more sensitive than the disc assay or Difco disc assay.

TABLE 10. Detection of penicillin in milk by the disc assay, Difco disc assay, and Charm test following intramammary infusion of 1 x 10^5 units of procaine penicillin G.

	Type of antibiotic assay[a]				
			Charm test		
Item	DA	DDA	0.025	0.005	0
Mean withdrawal					
time (h)	30.81[b]	36.63[b]	30.73[b]		
range (h)	10-48	10-58	10-58		
No. of milkings	2-5	2-5	2-5		
time (h)			30.75[c]	42.94[d]	53.0[e]
range (h)			10-58	24-72	24-96+
No. of milkings			2-5	2-6	3-7+

[a]DA = Disc assay, DDA = Difco disc assay.

[bcde](P<.05) Means with the same superscript are not significantly different.

Table 11 data show the comparison of the relative sensitivity of the disc assay, Difco disc assay, and Charm test methods for detection of penicillin in raw milk. The total number of positive tests for the presence of penicillin as detected by the Charm test at 0 level was 78. The disc assay and Difco disc assay detected

penicillin in 63% and 76%, respectively of the milk samples in which penicillin was detected by the Charm test at 0 level. The Difco disc assay based on the data was more sensitive than the disc assay or the Charm test at 0.025 level. It is much simpler than the disc assay, gives color and zone of inhibition, and could be readily adapted to the dairy laboratory, as it utilizes the basic disc assay technique which is familiar to technicians presently engaged in antibiotic assay. Additional studies should be undertaken to improve the distinct color identification, sensitivity, and speed.

TABLE 11. Comparison of the relative sensitivity between the disc assay, Difco disc assay, and Charm test for detecting penicillin in raw milk.

Type of treatment	Type of antibiotic assay[a]				
			Charm test		
	DA	DDA	0.025	0.005	0
	Number of positive tests				
Intramammary infusion	49	59	51	67	78
Sensitivity as % of Charm test at 0 level	63	76	65	86	100

[a]DA = Disc assay, DDA = Difco disc assay.

Out of 5200 commercial raw milk samples, Ginn et al. (21) detected 0.23% positive antibiotic samples by the disc assay and 0.92% by the Difco disc assay. They found the Difco disc assay to be simple, fast, and more sensitive than any currently used method for penicillin detection (21). The Charm test at 0.025 and 0.005

level detected penicillin in 65% and 86%, respectively of the milk samples in which penicillin was detected by the Charm test at 0 level. For testing tank truck supplies or storage tank milk, Charm test sensitivity (86%) would be a distinct advantage over the Difco disc assay (76%) or disc assay (63%). Perhaps the Charm test should be used only for tanker lots of commingled milk where the greater sensitivity and the speed (15 min) would be an advantage, while the Difco disc assay could be used for farm bulk tanks. A testing program with the Charm test would enable a plant to avoid commingling of contaminated milk into the silo, measuring after the fact, as is now done with the standard tests.

On the basis of the results obtained in this comparison of the relative sensitivities of the three antibiotic tests, a larger number of positive samples would be anticipated if the Charm test were used to assay milk samples instead of the disc assay or Difco disc assay. It is also conceivable that currently designated withholding times might be modified. In addition, an assay with the Charm test is more sensitive, quicker (15 min), and simpler with no more highly specialized microbiological techniques required than the disc assay or the Difco disc assay. Adoption of this method should be considered only after all the ramifications are considered even though the greater sensitivity would undoubtedly mean that more milk would be condemned. The extreme variation noted in the duplicate samples was very high, which is a negative attribute of this test. On the other hand the Difco disc assay exhibits

potential as a quantitative procedure. The procedure requires no special equipment or media. It is a simple technique requiring 2 h and 50 min, the standard penicillin discs and cultures of the test organism are readily available from commercial sources. The variation on duplicate samples is minimal.

SUMMARY

Milk samples from thirty-one fresh cows that received intrauterine infusions of three different antibiotic mixtures 3 days after parturition were analyzed for the presence of antibiotic residues using the disc assay and Charm test. The cows were divided into two groups, one group of cows with retained placentas and those without retained placentas. Withdrawal times for milk from the treated cows were determined with the disc assay method at one level and the Charm test at three levels of sensitivity.

The Charm test detected penicillin residues in the milk samples from cows that received intrauterine infusion for a significantly ($P<.01$) longer period than the disc assay. No significant difference ($P>.05$) was observed between disc assay and Charm test at 0.025 level, but good correlation was noted between them. Also, no significant difference ($P>.05$) was observed between cows with retained placenta and cows without retained placenta and the three different antibiotics that were infused into the uterus.

Eight healthy lactating cows were treated with 1×10^5 units of procaine penicillin G via intramammary infusion and the milk samples were analyzed for the presence of penicillin residues by the disc assay, Difco disc assay, and Charm test. Withdrawal times for the milk samples obtained from each treated cow were determined with each of the three antibiotic tests.

The disc assay and Difco disc assay methods detected penicillin in only 63% and 76%, respectively of the milk samples in which

penicillin was detected by the Charm test at 0 level. The Charm test at 0.025 and 0.005 level detected penicillin in 65% and 86%, respectively of the milk samples in which penicillin was detected by the Charm test at 0 level. Also, the Charm test detected penicillin residues for a significantly longer ($P<.01$) time than either the disc assay or Difco disc assay in milk following intramammary infusion. In all cases, the manufacturer's recommended withdrawal time of five milkings (60 h) was exceeded by at least two milkings when the milk samples were checked by the Charm test. However, the disc assay and Difco disc assay did not detect residues beyond the recommended withdrawal time of five milkings (60 h). Detectable penicillin crossover from treated to untreated quarters was observed with each of the three tests, especially with the more sensitive Charm test.

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APPENDIX TABLE 1. Analysis of variance for antibiotic concentrations in milk due to various factors following intrauterine infusion.

Source of variation	Degrees of freedom	Mean square
Placenta (P)	1	15.90
Treatment (T)	2	566.82
P x T	2	490.20
Tests (N)	3	7715.75**
P x N	3	150.12
T x N	6	179.80
P x T x N	6	105.74

**Significant (P<.01).

APPENDIX TABLE 2. Analysis of variance of penicillin concentrations in milk due to various factors following intramammary infusion.

Source of variation	Degrees of freedom	Mean square
Foreward to Rear (F)	1	128.68
Treatment (T)	1	51758.70**
F x T	1	238.40
Tests (N)	4	2798.60**
F x N	4	33.25
T x N	4	41.058
F x T x N	4	39.12

**Significant (P<.01).